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Review

RNA-dependent RNA polymerase encoded by hepatitis C virus: biomedical applications

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Abstract. The hepatitis C viruses (HCVs) are a group of small enveloped RNA viruses that have been viewed as a leading cause of chronic hepatitis in humans. Infections by HCV represent a serious global health problem, because millions of people worldwide are infected and no efficient treatment is available at the present time. Since HCV was identified in 1989, considerable effort has been devoted to the discovery and development of novel molecules to treat HCV-related diseases. One of the approaches is the development of novel inhibitors that interrupt the normal functions of HCV NS5B, an RNA-dependent RNA polymerase essential to HCV replication. This review summarizes recent advances in the biochemical and structural understanding of HCV NS5B polymerase as well as in the development of antiviral agents targeting this important enzyme.

Key words. HCV; viral RNA-dependent RNA polymerase; polymerase inhibitor; antiviral therapy.

Introduction

Hepatitis C virus (HCV), the etiologic agent responsible for non-A, non-B hepatitis in humans [1], is a leading cause of chronic liver diseases. Chronic HCV infections can proceed into the development of liver cirrhosis, hepatocellular carcinoma, and liver failure [2]. Over 170 million people worldwide are estimated as being infected by HCV [3]. The actual number of infected individuals might be higher since HCV infection is usually asymptomatic. Therefore, HCV infection has been viewed as a growing threat to human health worldwide.

HCVs are a group of single-stranded RNA viruses that belong to the Flaviviridae family [4]. All HCVs possess a positive-sense RNA genome of approximately 9600 nucleotides [4]. The HCV genomic RNA contains a single open reading frame which encodes a polyprotein precursor of ~3010–3033 amino acids [1]. The polyprotein precursor is further processed by cellular signal peptidase and virally encoded proteases to generate at least ten mature viral proteins/enzymes, in the order of core-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B [reviewed in refs 5, 6]. As summarized in figure 1, the first three proteins encoded by the HCV genome are structural: the nucleocapsid protein known as the core, and two glycosylated envelope proteins termed E1 and E2. Downstream of the structural proteins is the ~7-kDa membrane-associated protein, termed p7, with unknown function. NS2 to NS5B are the putative non-structural proteins that are essential to viral genome replication. For example, NS2 and part of NS3 define a unique proteolytic activity that selectively cleaves the NS2/3 junction [7, 8]. NS3 is multifunctional: its N-terminal ~180 amino acids encode a serine protease responsible for cleavage of NS3/4A, NS4A/4B, NS4B/ 5A, and NS5A/5B junctions [see ref. 9 for review]; its C-terminal 451 amino acids define an ATP-dependent helicase unwinding activity that might be required for viral genome replication [see ref. 10 for review]. NS4A is a small protein that serves as a cofactor

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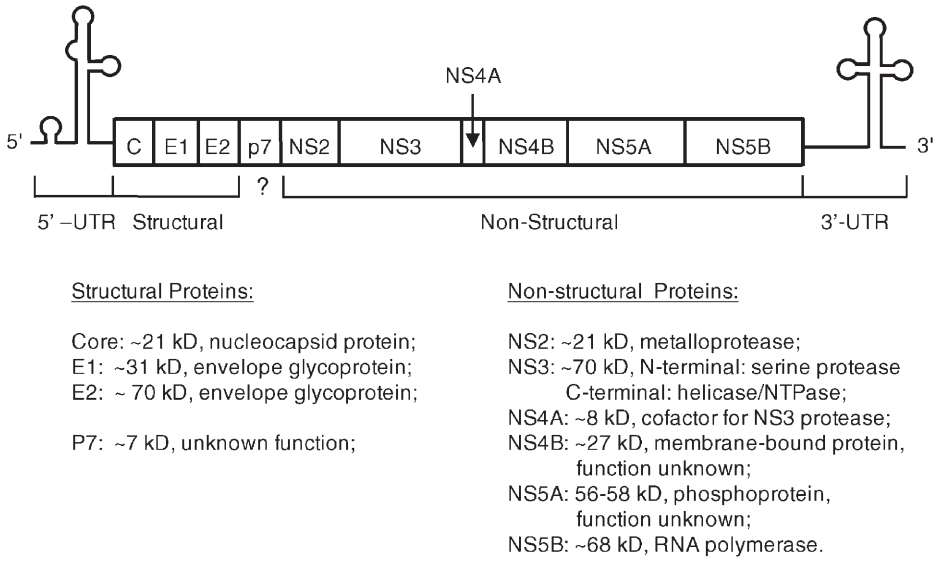


Figure 1. Schematic representation of the HCV genome and viral proteins. The boxed area corresponds to the open reading frame. Stem-loop structures represent the 5′- and 3′-untranslated regions (UTR). The function and molecular weight (in kD) of each viral protein are also listed.

for NS3 protease activity [11, 12]. NS4B is a membrane-bound protein and NS5A is a phosphorylated protein in infected cells. Although their functions have not been clearly elucidated [reviewed in ref. 13], NS5A has recently been shown to bind and regulate NS5B (see below). The last protein encoded by the viral genome is designated NS5B, an RNA-dependent RNA polymerase that is responsible for viral RNA synthesis [4, 14].

For years, treatment of HCV infections has been limited to interferon-α (IFN-α) alone or in combination with ribavirin. However, these treatments have demonstrated unsatisfactory efficiency and are associated with severe side effects [reviewed in ref. 15]. Therefore, since HCV was identified in 1989, intensive efforts have been directed to the development of effective prevention and treatment of HCV-related infections. The development of prophylactic vaccines has been difficult and no significant breakthrough has been made to date, due in part to the existence of large numbers of HCV genotypes, subtypes, and quasispecies [reviewed in ref. 16]. As for the development of efficient treatments, progress has been made through different approaches. The first example is the recent approval of pegylated IFN-α isoforms, generated through modification of recombinant IFN by polyethylene glycol, to be used for treatment of HCV infections. Compared to the unmodified form, the pegylated-IFN-α showed improved pharmacokinetic profiles such as an increased half-life in the circulation and reduced immunogenicity, which permit lower and less frequent dosage and thus fewer side effects [reviewed in refs 17–19]. The second example is the potential treatment of HCV infection using inhibitors targeting cellular inosine monophosphate dehydrogenase, an essential enzyme catalyzing de novo biosynthesis of guanosine nucleotides. One of such inhibitors, designated VX-497, demonstrated both antiviral and anti-inflammation activity and is currently being evaluated in phase II trials for potential use as an anti-HCV agent [20]. A third example is the antiviral approach of targeting the HCV RNA genome. Molecules including an antisense oligonucleotide and a catalytic ribozyme were advanced into clinical trials for further investigation [reviewed in refs 18, 21]. Therefore, there is hope that an effective antiviral agent that is better than the current treatment options will emerge from these various approaches in the near future.

Parallel to the major advances mentioned above, development of antiviral agents targeting HCV proteins/enzymes is another approach to combat HCV infections. Of the ten proteins encoded by the HCV genome (fig. 1), the NS3 protease and the NS5B polymerase have been viewed as the most attractive targets for antiviral chemotherapy due to the clinical success of protease and polymerase inhibitors for other pathogenic viruses, including HIV and HBV [17, 22]. Particularly for the HCV NS5B polymerase, great attention has been given to this enzyme, due not only to its essential function in the viral replication process but also to the lack of known counterparts in host cells. Since the enzymatic activity of NS5B was first demonstrated in 1996 using purified recombinant protein [23], numerous groups have reported biochemical and structural characterization of this viral enzyme. More recently, compounds with diversified structures inhibiting the HCV polymerase activity have been identified and developed. This review focuses on recent progress in the development of antiviral agents that specifically inhibit this enzyme, and summarizes re-

search information regarding NS5B to promote a better understanding of this unique antiviral target.

Generation of recombinant HCV NS5B polymerase

The NS5B protein encoded by the HCV genome was predicted to be an RNA-dependent RNA polymerase due to the presence of the hallmark sequence – GDD – along with five conserved motifs (A to E) also seen in other viral RNA-dependent RNA polymerases [4, 14]. As a key component involved in HCV RNA genome replication, this enzyme catalyzes synthesis of a complementary minus-strand RNA using the viral genome RNA as a template, and the subsequent synthesis of genomic plus-strand RNA from this minus-strand RNA template. Due to the low efficiency of HCV replication in cell culture systems, a recombinant protein expression approach is the only efficient mean to obtain active NS5B polymerase at the present time.

The first recombinant NS5B protein described in the literature is the expression of a full-length NS5B of HCV genotype 1b in insect cells using a baculovirus expression vector [23]. After extraction and solubilization using non-ionic detergent in combination with high salt and high glycerol concentrations, this full-length, untagged NS5B was purified by sequential chromotographic columns [23, 24]. Purified NS5B could catalyze RNA synthesis from both HCV RNA genome or homopolymeric RNA templates [23, 24]. Shortly thereafter, a histidine-tagged NS5B protein was also expressed in insect cells [25]. Compared to the untagged NS5B, this tagged form could be purified to near homogeneity using a single affinity column [25].

The generation of active HCV NS5B in bacterial cells has been reported by several groups. For example, an enzymatically active NS5B protein fused to the glutathione S-transferase (GST) has been produced in *Escherichia coli* cells [26]. Other tagged NS5B protein forms, carrying a histidine cluster as an affinity tag at either the N or C terminus of the protein, have also been over-expressed and purified in bacterial cells [27, 28]. In addition to these tagged HCV NS5B proteins, several groups have described expression of non-fusion NS5B proteins, either full-length or C-terminal truncation, in bacterial cells [29–32].

Unlike the HCV NS3 serine protease, generation of active HCV NS5B using recombinant systems has been difficult: the first active recombinant NS5B was reported approximately 7 years after the HCV genome was identified [23]. A large portion of NS5B isolates, when expressed in recombinant systems, were inactive or weakly active in the in vitro RNA polymerase assay. The reason, at least in part, may be that the presence of HCV quasispecies results in a mixed pool of viruses that carry divergent NS5B sequences with varied RNA polymerase activity [33]. For example, NS5B proteins containing only three amino acids different from an active NS5B were enzymatically inactive [33]. Therefore, the amino acid sequences of NS5B play an important role in generating active enzyme. Several other factors that affect the production and catalytic efficiency of recombinant NS5B proteins have also been discussed. For example, attachment of an affinity tag at the N terminus of the NS5B might affect its enzymatic activity or protein solubility [25, 28]. Early studies revealed that NS5B with an N-terminal histidine tag was not only less expressed but also less active compared to the C-terminal histidine-tagged NS5B proteins [25, 28]. Furthermore, the HCV NS5B protein contains a hydrophobic region at its C terminus responsible for membrane association [26, 34]. Deletion of this hydrophobic region was not deleterious to NS5B catalytic activity, and in fact, the truncated version showed higher catalytic efficiency than the intact protein [28, 29]. Consequently, expression of a C-terminal truncation form of NS5B has been found to be easier than that of the full-length protein [26, 28] and more soluble truncated NS5B proteins can be generated through this approach.

Structural analysis of HCV NS5B polymerase

HCV NS5B polymerase contains a total of 591 amino acids. Analysis of the HCV NS5B protein sequence revealed the existence of five conserved motifs, designated motif A through E, which are found in other RNA-dependent RNA polymerases [14, 35]. Unlike poliovirus 3D RNA polymerase, HCV NS5B contains a hydrophobic C terminus composed of 21 amino acids (fig. 2). Although this domain was not essential for the catalytic activity of HCV NS5B in cells [26], deletion of this fragment deprives the NS5B protein of the ability to associate with endoplasmic reticulum membrane and alters NS5B protein localization in vivo [26, 34]. This region has been suggested as a membrane-anchoring domain and is involved in the formation of membrane-associated replication complexes [34]. In contrast, deletions at the N terminus are indeed detrimental to NS5B polymerase activity as discussed below [25]. Further mutational analysis has suggested that the catalytic core of HCV NS5B is contained in the N-terminal ~540 amino acids [29] (fig. 2).

The availability of large quantities of purified HCV NS5B proteins has permitted detailed structural analyses. The three-dimensional crystal structure of this protein has been determined by several groups [35–37]. Besides minor differences in primary sequence and C-terminal truncation length, these structures are superimposable. In all structures reported, the HCV NS5B protein displays the fingers, palm, and thumb subdomains characteristic

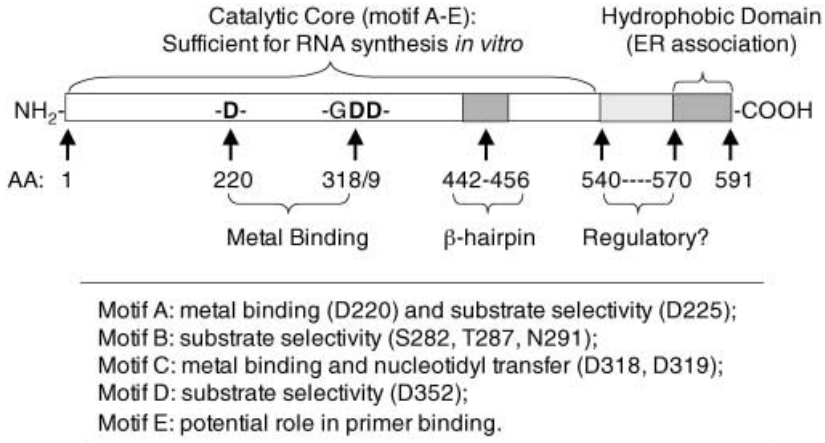


Figure 2. Schematic representation of the HCV NS5B protein with known functional groups. Shown is the NS5B polymerase containing 591 amino acids (AA). Key domains/motifs as well as the key residues involved in metal/nucleotide binding are highlighted. Residues 442–456 form a β-hairpin believed to be important in guiding RNA initiation.

of all known RNA and DNA polymerases as shown in figure 3. In addition, the locations of the five conserved motifs have also been identified from these structural studies [35–37]. Although none of these structures was solved with RNA or metal ions bound to the active site, Bressanelli and colleagues [38] recently identified a specific GTP-binding domain. This binding pocket, 30 Å away from the active site, may serve as a conformational switch between initiation and elongation. In addition, the residues involved in binding of RNA and metals have been highlighted by superimposition with the ternary complex of HIV reverse transcriptase [39]. For example, two aspartates, Asp220 and Asp318, were found to be the key residues for nucleotide metal binding, consistent with the biochemical data obtained from site-directed mutagenesis of HCV NS5B [25].

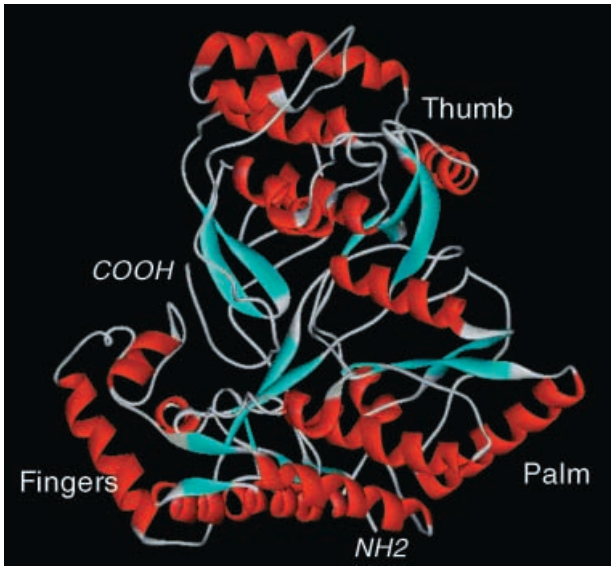


Figure 3. Crystal structure of the HCV NS5B polymerase. The first 570 residues from the N terminus are shown in the ribbon conformation at 2.1-Å resolution. The thumb, finger, and palm subdomains are common to all known polymerases. α helices and β strands are shown in red and cyan, respectively. Positions of the N and C terminus of the protein are marked as NH2 and COOH, respectively [for more details see ref. 56].

Several interesting structural features were revealed through analysis of the HCV NS5B crystal structure. Among the unique features, HCV NS5B polymerase includes an encircled active site with an overall globular shape instead of the typical U shape found in other polymerases [35–37]. Such an encircled active site is due to the extensive interactions between the fingers and the thumb subdomains, which close the gap between the two subdomains. The N-terminal residues, Ala9–Thr41, are extensively involved in these interactions, explaining the essential roles of N-terminal residues to NS5B polymerase activity [25]. As a result, HCV NS5B is unlikely to open up upon binding of the RNA template/primer complex [35–37], as in the case for HIV reverse transcriptase [39].

Another novel structural component unique to HCV polymerase is a β-hairpin found in the thumb subdomain. This β-hairpin, composed of ~15 amino acids representing residues 442–456, protrudes into the RNA-binding cleft [35–37]. One of the potential roles of this unique structure might be to recognize a specific sequence of HCV genomic RNA for correct initiation of replication, as in the case of the specificity loop identified in T7 RNA polymerase [40]. Supporting this hypothesis, deletion of this loop from the NS5B protein can lead to increased internal initiation, suggesting that this β-hairpin serves as a gate ensuring a correct RNA initiation from the 3′ terminus of the RNA template [41]. Of interest is that poliovirus 3D polymerase does not have such an analogous structure, coinciding with the fact that 3D polymerase

Table 1. C-terminal consensus amino acid sequence of HCV NS5B polymerase.

AA number	535						540						545					550
Consensus	**T**	**K**	L	K	**L**	**T**	**P**	I	P	A	A	S	Q	**L**	**D**	**L**	**S**	G	**W**
Conservation (%)	100	100	93	98	100	100	100	78	84	79	99	70	54	100	100	100	100	67	100

AA number	555						560						565					570
Consensus	**F**	V	A	G	Y	S	**G**	G	D	I	Y	H	**S**	L	**S**	R	A	**R**	P	**R**
Conservation (%)	**100**	65	81	99	83	71	**100**	95	99	97	98	99	**100**	60	**100**	78	98	**100**	95	**100**

Sequence alignment of HCV NS5B proteins from 134 different genotypes and subtypes was performed using the CLUSTAL W (1.81) program. Shown is the fragment representing amino acids 532–570 upstream of the C-terminal membrane-anchor domain of HCV NS5B (amino acid position is labeled AA number). Consensus residues (middle line) are expressed in the single-letter code. The conservation percentage is obtained from sequence comparison.

initiates viral RNA replication through priming the viral 3AB protein, a mechanism that is unique to polio and related viruses [42].

In addition, the C-terminal segment containing residues 532–570, immediately upstream of the membrane anchorage domain, seems to be unique to HCV NS5B polymerase. Amino acid sequence alignment of HCV NS5B proteins from different genotypes and subtypes reveals several completely conserved residues in this region (table 1). Interestingly, this fragment, together with the downstream membrane anchor domain, is not present in other polymerases including poliovirus 3D polymerase and HIV reverse transcriptase [14, 35]. Although truncated NS5B proteins with a deletion of this region exhibit RNA-dependent RNA polymerase activity [25, 29], this fragment, buried in the putative RNA duplex-binding cleft, has been predicted to play a role in regulating HCV NS5B polymerase activity in vivo [36] (fig. 2).

Catalytic specificity of HCV NS5B polymerase

Even though HCV NS5B polymerase activity was first reported over 6 years ago [23], the catalytic mechanism of NS5B at a molecular level is largely unknown. Our current understanding of this enzyme relies mainly on characterization of recombinant NS5B proteins using various in vitro biochemical assays measuring the overall incorporation of labeled nucleotides. A typical NS5B polymerase assay described in the literature measures the incorporation of radiolabled nucleotides, i.e., RNA synthesis, in the presence of divalent cation and an RNA template, which can be either a homopolymeric RNA or HCV RNA of various sizes, in the absence or presence of appropriate primers.

HCV NS5B is indeed an RNA-dependent RNA polymerase: it does not contain DNA-dependent RNA polymerase activity or RNA-dependent DNA polymerase (reverse transcriptase) activity because it cannot utilize DNA as template or deoxynucleotide as substrate [26, 32]. However, HCV NS5B can transfer nucleotidyl substrates to a DNA primer hybridized to the RNA template for RNA elongation [26, 32]. The RNA synthesis catalyzed by HCV NS5B was originally believed to be an elongation reaction via a copy-back mechanism or using an appropriate primer, through which nucleotidyl residues could be transferred to the 3′-hydroxyl group of the last nucleotide of the primer that was hybridized to the template [23]. Recently, several groups reported that HCV NS5B was also able to catalyze de novo RNA initiation [27, 33, 43–45]. In this regard, purified HCV polymerase was able to initiate RNA synthesis in the absence of any cofactors by recognizing pyrimidine residues, i.e., cytidylate or uridylate, present in the templates, a mechanism that is expected to occur in vivo during viral genome replication [43–46].

The catalytic efficiency of the HCV NS5B enzyme was directly associated with the nature of the templates used in the assay. For example, purified NS5B could synthesize RNA using polyC and polyA as templates but did not do so efficiently using polyU or polyG RNAs [26, 32, 47]. As expected, this enzyme could also replicate the entire HCV genome or its genome fragments through both copy-back and/or de novo initiation mechanisms [27]. In addition, the catalytic efficiency of HCV NS5B was affected by the concentration and length of the RNA template and primer. For example, the optimized primer length for elongation has been determined to be between eight to ten nucleotides [32, 45]. Ribonucleotides such as GTP were found to stimulate the catalytic activity of NS5B in vitro [48]. Although the underlying mechanism is not yet fully understood, GTP has been recently proposed to act as an allosteric regulator, through binding to a shallow pocket at the surface of the enzyme [38].

Different laboratories have determined kinetic parameters for the NS5B RNA polymerization reaction using both homopolymeric RNA and short RNA templates derived from the 3′-terminal HCV genome. Based on steady-state kinetic analysis, the catalytic efficiency determined for HCV NS5B polymerase was in the range of 200–2000 pmol/h^{-1}/μg^{-1} when using homopolymeric RNA as template [32, 47], while NS5B processivity was ~10 pmol/h^{-1}/μg^{-1} when HCV 3′-UTR-like RNA was used as template [49]. This specific activity seems to be

lower than reported for HIV reverse transcriptase and other viral polymerases [28, 50]. The observed low efficiency might be due, at least in part, to assay conditions as described above and/or a large portion of inactive NS5B protein mixed in the enzyme preparation, as demonstrated recently by Carroll and coworkers [51]. In addition, formation of non-productive enzyme-RNA-binding complexes, as observed for poliovirus 3D polymerase [52], could also contribute to the low catalytic efficiency of HCV NS5B.

Like other known polymerases, HCV NS5B also requires divalent cations for catalysis. The most preferred metals have been identified as Mg^{2+} at concentrations of ~5–10 mM or Mn^{2+} at lower concentrations (<1 mM). In contrast to the role of Zn^{2+} for poliovirus 3D polymerase activity, this metal was found to inhibit HCV polymerase with an IC_{50} determined as 60 µM [28]. The involvement of divalent cations in the reactions catalyzed by HCV NS5B can be explained by the two-metal ion mechanism established for other DNA and RNA polymerases [53]. As mentioned above, these metals, together with several conserved aspartates, are responsible for coordination of nucleotide binding to the enzyme (fig. 2). HCV NS5B prefers a neutral pH in vitro, and has demonstrated highest activity at temperatures ranging from 22 to 32 °C with a significantly decreased efficiency when tested at 37 °C, raising questions as to how the viral genome can be effectively replicated at physiological conditions.

Oligomerization and cooperative activity of HCV NS5B polymerase

Similar to the poliovirus 3D polymerase [54, 55], HCV NS5B has been found to form functional oligomers and to catalyze cooperative RNA synthesis in vitro [56, 57]. Oligomerization of NS5B has been detected both in vitro and in cells as demonstrated by gel filtration, chemical cross-linking, temperature sensitivity, and analyses using yeast two-hybrid and transient expression in mammalian cells [56, 57]. More importantly, oligomerization of NS5B can affect its catalytic efficiency: the enzyme demonstrated cooperative RNA synthesis in a manner similar to that described for the poliovirus 3D enzyme. Mutational analyses suggested that several residues including Glu18 and His502 were critical for NS5B oligomerization [57], as well as for catalytic activity. In addition, several motifs and domains condensed at two extensive interfaces of the HCV NS5B molecules were reported to be likely implicated in NS5B protein-protein interaction [56]. For poliovirus 3D polymerase, oligomerization is essential for viral replication and infection [58]. Whether NS5B oligomerization has similar impacts on HCV replication remains to be determined.

Table 2. Summary of the TNTase activity reported for HCV NS5B.

NS5B	Expression	RNA template	TNTase activity	Ref.
1a	insect cells	X region, U_{12}	yes	23
1b	insect cells	X region	yes	24
1b	insect cells	U_{12}	no	25
1b	*E. coli* cells	X region, A_{20}, U_{20}	no	27
1b	*E. coli* cells	A_{12}, U_{12}	no	32
1b	*E. coli* cells	RNA_{40}	no	59
1b	insect/*E. coli* cells	BVDV-RNA	yes	60

X region, the last 98 nucleotides of the 3′ UTR of the HCV RNA genome; A_{12} or A_{20}, 12-mer or 20-mer polyadenine; U_{12} or U_{20}, 12-mer or 20-mer polyuridine; RNA_{40}, 40-mer synthetic heteropolymeric RNA; BVDV-RNA, derived from the 3′end of bovine viral diarrhea virus (–)-strand RNA.

Terminal nucleotidyl transferase activity of HCV NS5B

In addition to the RNA-dependent RNA polymerase reactions catalyzed by HCV NS5B, terminal nucleotidyl transferase activity (TNTase), the ability of an enzyme to catalyze addition of a nucleotide to the 3′ end of the input RNA, was originally claimed as an intrinsic property of HCV NS5B expressed in insect cells [23]. However, this activity was later shown to result from contaminants present in the enzyme preparation [25]. As summarized in table 2, this conclusion was further confirmed by several groups using various HCV NS5B proteins expressed and purified from bacterial cells [25, 27, 32, 59]. More recently, Kao and colleagues [60] reported that HCV NS5B demonstrated TNTase activity in a template-dependent manner, supporting the original observation. Therefore, the verdict is still out on whether the TNTase activity is an intrinsic property of HCV NS5B, or results from contaminating proteins present in the NS5B preparations. If HCV NS5B indeed catalyzes terminal transfer of nucleotides to RNA and if this function is essential for HCV genome replication, this activity could serve as an additional target for antiviral intervention.

Other biochemical properties of HCV NS5B

Similar to other polymerases, HCV NS5B protein can bind to various nucleotides and RNAs [32, 46, 47]. Of note is that the binding efficiency of NS5B toward ssRNA differs from that for dsRNA [59]. To ensure RNA duplex or dsRNA binding, HCV NS5B might have to go through a modest conformational change; in particular, the β-hairpin, protruding into the RNA-binding site as described above, might need to move out of the way so that a dsRNA molecule could fit in the RNA-binding cleft [36]. In addition, HCV NS5B expressed in Huh-7 cells

was recently shown to be associated with the endoplasmic reticulum membranes [34, 61] and to form a complex with human ribosomal RNAs, implicating a potential mechanism of coupling HCV genome replication and translation in the HCV life cycle [62].

Specific binding of NS5B protein to the HCV genome, particularly to the 3′ UTR of the viral RNA, has been studied. NS5B alone was recently shown to recognize specific structure elements from the 3′ UTR of the minus-strand RNA of HCV [63], and the conserved stem-loop structure within the 3′ UTR region of the plus-strand RNA [64]. In infected cells, these stem-loop elements might guide NS5B to bind at the appropriate position to initiate a correct replication of viral RNA. Consistent with this hypothesis, the unique β-hairpin of NS5B has been postulated to help modulate such a sequence-specific recognition during the RNA replication process, similar to the role of the specificity loop identified in T7 RNA polymerase [40]. Therefore, a specific interaction between this β-hairpin and the 3′ UTR should be anticipated.

In addition to RNA binding, several proteins encoded by the HCV genome might interact with the NS5B protein. Early studies suggested that complex formation might happen among NS5B, NS3, and NS5A in vitro [65, 66]. Recently, a more detailed analysis was performed on the complex formed between HCV NS5B and NS5A proteins. Two regions of NS5A, each containing ~60 amino acids, were found to be responsible for NS5B binding [67]. Interestingly, the NS5B RNA-dependent RNA polymerase activity was enhanced by NS5A at a low concentration (<1:10 ratio to NS5B) but became inhibitory at higher concentrations [67]. Whether this interaction exists in HCV-infected cells as part of the replication complex is not yet clear. Nevertheless, a modulating role of NS5A in HCV replication, through regulating NS5B activity, has been hypothesized on the basis of these observations. In addition, interaction of HCV NS5B with cellular proteins has also been reported by different groups [68, 69]; however, the physiological relevance of these interactions is unclear at the present time.

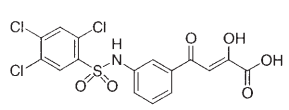
The HCV NS5B protein might undergo post-translational modification such as phosphorylation [70]. NS5B can be phosphorylated on certain serine and threonine residues in cells and this modification could positively regulate its catalytic efficiency, as demonstrated in the in vitro RNA polymerase assays [71]. On the other hand, HCV NS5B proteins that were expressed in bacterial cells, and thus lacked potential phosphorylation, also demonstrated RNA synthesis activity [26–28, 32], arguing that NS5B phosphorylation was not essential for NS5B enzymatic activity. Apparently, the physiological role of NS5B phosphorylation merits further investigation.

Development of inhibitors targeting HCV NS5B polymerase

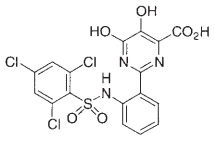
As the knowledge regarding HCV NS5B structure and function accumulates, development of specific NS5B enzyme inhibitors has been greatly promoted. In general, NS5B is not sensitive to most nucleotide analogs that inhibit other viral polymerases; it was not inhibited by most of the known inhibitors for DNA-dependent DNA polymerases or reverse transcriptases [24, 28, 32]. In addition, cerulenin and gliotoxin, originally identified as inhibitors for other viral RNA polymerases such as the poliovirus 3D polymerase, inhibited NS5B activity with only moderate potency [17, 28, 47, 72]. Certain positively charged polymers such as heparin and polylysine were shown to inhibit HCV NS5B polymerase [29, 32]. Although these reagents have been used to study the catalytic mechanism of the enzyme, their potential use as antiviral agents seems to be limited.

Specific inhibitors for HCV polymerase were not identified until recently. A group of diketobutanoic acid derivatives were first claimed as inhibitors for HCV NS5B polymerase. Over 200 compounds including alkyl-, phenyl-, pyrrole-, and thiophene-substituted diketoacids were evaluated against HCV NS5B polymerase activity [73]. Of these, several phenyldiketoacids demonstrated low nanomolar IC_{50} values (scheme 1). These compounds interfere with the binding of phosphoryl groups of the nucleotide substrates at the active site of the viral polymerase, and therefore inhibit the formation of phosphodiester bonds catalyzed by the enzyme [74]. More recently, the same group reported a series of 2-aryl-4,5-dihydroxy-carboxypyrimidines, similar to diketoacids as seen in scheme 1, also inhibiting HCV NS5B polymerase activity [75]. Certain compounds of this class were claimed to have IC_{50} values at or below micromolar concentration (scheme 1). Worth noting is that diketoacids were reported to have reasonable activity in the cell-based HCV subgenomic RNA replicon assay [74]; however, whether or not these inhibitors have advanced into clinical trials is not known.

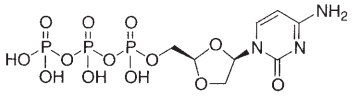
Despite the wide use of nucleoside analogs for treatment of hepatitis B virus and HIV infections, few nucleoside analogs have been identified as potent inhibitors of HCV polymerase. One group described the potential use of purine and pyrimidine analogs to treat HCV infection through blocking the viral polymerase activity [76]. Some of the triphosphate forms of the claimed cytosine and guanine analogs showed a low micromolar range of IC_{50}s against HCV NS5B activity in vitro [76] (scheme 1). No further information on the development of these nucleoside analogs is available. Recently, HCV NS5B polymerase was reported as a mediator of the antiviral activity of ribavirin, a nucleoside analog that has been used as an antiviral agent to treat HCV infections [77, 78].



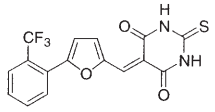
Diketobutanoic Acid
IC_{50} = 0.049 μM



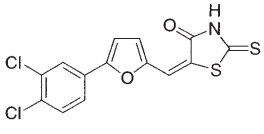
Dihydroxy-carboxypyrimidines
IC_{50} < 1 μM



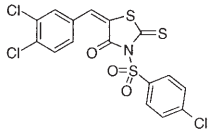
Nucleoside Analogs
IC_{50} = 7.7 μM



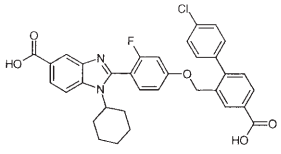
Barbituric Acid Derivatives
IC_{50} = 0.1-30 μM



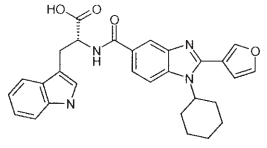
Rhodanine Derivatives
IC_{50} < 1 μM



Rhodanine Analogs
IC_{50} < 1 μM



Benzimidazole Derivatives
IC_{50} = 0.010 μM



Benzimidazole Derivatives
IC_{50} < 1 μM

Scheme 1. Summary of HCV NS5B Polymerase Inhibitors.

Hopefully, a detailed understanding of the inhibition mechanism of ribavirin will help in the design of more selective and potent nucleoside analogs as anti-HCV therapy.

Rhodanine and barbituric acid derivatives have also been claimed as HCV polymerase inhibitors by different groups [79–81]. Inhibition potencies were quoted in micromolar or submicromolar ranges against purified NS5B polymerase (scheme 1). Mechanism studies showed that rhodanine derivatives or analogs inhibited HCV polymerase through the formation of a covalent bond with a conserved cysteine residue, Cys366, located in the catalytic center of NS5B polymerase [81]. The covalent bond formed between the enzyme and certain compounds was claimed to be reversible [81]. However, due to the nature of the interaction between rhodanines and the enzyme, special attention should be given to rhodanine derivatives with respect to potential toxicity.

More recently, benzimidazole derivatives have been identified as HCV polymerase inhibitors by different groups [82, 83]. Unfortunately, although inhibition of the viral enzyme by some of the claimed benzimidazoles has been reported in the nanomolar range, no data have been quoted regarding their potency in cell-based assays [82, 83]. The advancement of an oral formulation of a benzimidazole derivative into clinical trials has been reported, and information on clinical efficacy is anticipated soon.

Use of macromolecules to block the functions of HCV polymerase has also been explored. Several monoclonal antibodies that specifically recognize HCV NS5B polymerase have been characterized recently [84, 85]. Interestingly, one of these was found to inhibit HCV polymerase activity through blocking NTP, but not RNA, binding to the enzyme [85]. Hopefully, elucidation of the detailed interaction between monoclonal antibodies and

HCV polymerase may provide useful information for the development of small molecules as potent HCV polymerase inhibitors.

Conclusions

HCV is viewed as an emerging threat to human health worldwide and no effective prophylactic vaccines or satisfactory antiviral therapy are currently available for the prevention and/or treatment of HCV-related infections. The NS5B polymerase encoded by the HCV genome represents an attractive target for antiviral therapy. To date, nanomolar range inhibitors against HCV polymerase have been identified on the basis of a comprehensive understanding of the viral enzyme, and this may provide optimism that certain potent polymerase inhibitors will be advanced into clinical development in the near future.

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